OPTIMIZING GRAPE ROOTSTOCK PRODUCTION AND EXPORT OF INHIBITORS OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE ACTIVITY

Principal Investigator:
John M. Labavitch
Department of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Co-Principal Investigator:
Ann L.T. Powell
Department of Plant Sciences
University of California
Davis, CA 95616
alpowell@ucdavis.edu

Co-Principal Investigator:
Alan Bennett
Department of Plant Sciences
University of California
Davis, CA 95616
abbennett@ucdavis.edu

Co-Principal Investigator:
Daniel King
Dept. of Chemistry & Biochemistry
Taylor University
Upland, IN 46989
dnking@taylor.edu

Co-Principal Investigator:
Rachell Booth
Dept. of Chemistry & Biochemistry
Texas State University
San Marcos, TX 78666
rbooth@txstate.edu

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ABSTRACT
The CDFA Pierce's Disease (PD) and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel review in 2007 and subsequent RFPs have given top priority to delivery from grafted rootstocks of PD control candidates, including polygalacturonase-inhibiting proteins (PGIPs). PGIPs are plant proteins that inhibit pathogen and pest polygalacturonases (PGs). In this project, multiple PGIPs were evaluated for the efficiency of their inhibition of *Xylella fastidiosa* (*Xf*) PG. Fourteen candidate PGIPs have been chosen and predicted protein structure models were developed to identify interactions with and potential inhibition of *Xf*PG. PGIPs from pear, rice, and orange were determined to be the most likely PGIPs to effectively inhibit *Xf*PG. Recombinant protein expression systems have been developed for *Xf*PG and candidate PGIPs in grape and tobacco plants. Initial inhibition assays have shown that the pear fruit PGIP is a more effective inhibitor of *Xf*PG than the tomato PGIP, however both grape and pear PGIPs limit *Xf*PG symptom development in tobacco leaf infiltration assays. Evaluation of additional PGIPs is underway.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) uses a key enzyme, polygalacturonase (PG), to spread from the initial point of inoculation throughout the grapevine; this spread leads to Pierce's disease (PD) symptom development. Proteins called PG-inhibiting proteins (PGIPs) are produced by many plants and these PGIPs selectively inhibit PGs from bacteria, fungi, and insects. The PGIP expressed in pear fruit is known to inhibit *Xf*PG and limit PD development in inoculated grapevines which have been engineered to express the PGIP protein normally present in pear fruit. PGIP proteinss are secreted from cells and they are can travel across graft junctions. We are interested in identifying the PGIPs that best inhibit *Xf*PG and ascertaining how well, when this PGIP is expressed in transgenic rootstocks, it prevents PD development in grafted wild-type scions inoculated with *Xf*. We have modeled the protein structures of fourteen candidate PGIPs to predict how each of them physically interacts with *Xf*PG. We will combine this knowledge with *in vitro* and *in planta* assay results measuring the ability of the candidate PGIPs to inhibit *Xf*PG in grapevines. For these inhibition assays we are developing systems to generate high levels of active *Xf*PG and PGIPs. The best inhibiting PGIPs will be expressed in test grape rootstock germplasm and, after grafting, their ability to limit PD development in non-transgenic scions will be determined in vineyard settings.

INTRODUCTION
Xylella fastidiosa (*Xf*), the causative agent of Pierce's disease (PD) in grapevines, has been detected in infected portions of vines. Several lines of evidence support the hypothesis that *Xf* uses cell wall-degrading enzymes to digest the polysaccharides of plant pit membranes that separat the elements of the water-conducting vessel system, the xylem, of the vines. *Xf*'s cell wall degrading enzymes break down these primary cell wall barriers between cells in the xylem, facilitating the systemic spread of the pathogen. Recombinantly expressed *Xf* polygalacturonase (*Xf*PG) and β−1,4-endo-glucanase (EGase), cell wall degrading enzymes that are known to digest cell wall pectin and xyloglucan polymers respectively, have been shown to degrade grapevine xylem pit membranes and increase pit membrane porosity enough to allow passage of the bacteria from one vessel to the next (Pérez-Donoso *et al.*, 2010). *Xf* cells have been observed passing through degraded pit membranes without the addition of exogenous cell wall degrading enzymes, supporting the conclusion that the enzymes are expressed by *Xf* and allow its movement within the xylem by degrading the pit membranes (Labavitch and Sun, 2009). Roper *et al.* (2007) developed a PG-deficient strain of *Xf* and showed that the mutant bacterial strain was unable to cause PD symptoms; thus, the *Xf*PG is a virulence factor of the bacteria that contributes to the development and spread of PD. PG-inhibiting proteins (PGIPs) produced by plants are selective inhibitors of PGs and limit damage caused by fungal pathogens (*B. cinerea*; Powell *et al.*, 2000) as well as by insects (*Lygus hesperus*; Shackel *et al.*, 2005). Agüero *et al.* (2005) demonstrated that by introducing a pear fruit PGIP (*pPGIP*) gene (Stotz *et al.*, 1993) into transformed grapevines, the susceptibility to both fungal (*Botrytis cinerea)* and bacterial (*Xf)* pathogens decreased. This result implied that the pPGIP

provided protection against PD by inhibiting the *Xf*PG, reducing its efficiency as a virulence factor. In fact, recombinant *Xf*PG is inhibited *in vitro* by pPGIP-containing extracts from pear fruit (Pérez-Donoso *et al.*, 2010). In a key preliminary observation for the PD control approach investigated in this project, Agüero *et al.* (2005) demonstrated that transgenic pPGIP protein could be transported from transformed grapevine rootstocks, across a graft junction and into the grafted wild-type scions. pPGIP also has been shown to be transported from rootstocks across grafts into the aerial portions of tomato plants. The overall goal of the project is to develop transgenic grape rootstock lines that express PGIPs that effectively reduce the virulence of *Xf*. The project is designed to compare potential *Xf*PG inhibiting properties of PGIPs from a wide variety of plants in order to identify specific PGIPs that optimally inhibit the virulence factor, *Xf*PG. The goal is to express these PGIPs in grape rootstocks to provide PD protection in grafted scions. The expression of PGIPs in grape rootstocks will utilize transformation components with defined intellectual property (IP) and regulatory characteristics, as well as expression regulating sequences that result in the maximal production of PGIPs in rootstocks and efficient transport of the proteins through the graft junctions to the aerial portions of vines so that *Xf* movement is limited in infected scion tissues.

OBJECTIVES

1. Define a path for commercialization of a PD control strategy using PGIPs, focusing on IP and regulatory issues associated with the use of PGIPs in grape rootstocks.
   a. Evaluate IP and licensing status of the plant expression construct components for the PGIP-based rootstock strategy (Year 1)
   b. Assemble grape transformation vectors utilizing PIPRA vectors with defined IP characteristics (Year 2)
2. Identify plant PGIPs that maximally inhibit *Xf* PG.
   a. Use existing pear PGIP-expressing grapes, test PD susceptibility of normal scions grafted to PGIP-expressing and -exporting roots (Years 1 and 2)
   b. Identify plant PGIPs that are efficient inhibitors of *Xf*PG (Year 1)
   c. Express PGIPs in Arabidopsis thaliana and test for optimal inhibition of *Xf* PG (Years 1 and 2)
   d. Optimally express *Xf* PG, using recombinant protein expression systems (Year 1)
   e. Model PGIP and *Xf* PG interactions to identify optimal PGIPs for PD defense (Years 1 and 2)
3. Assemble transcription regulatory elements, *Xf*-inducible promoters and signal sequences that maximize PGIP expression in and transport from roots.
   a. Make transformed grape lines using the best PGIP candidates, promoters etc. (Years 2 and 3)
4. Create PGIP-expressing rootstocks and evaluate their PD resistance.
   a. Molecular analysis of putative marker free transgenic grape plants (Year 3)
   b. Evaluate transgenic grape lines for optimal expression and export to scions of selected PGIPs (Year 3)
   c. Evaluate transgenic lines for susceptibility to *Xf* (Year 3)

RESULTS AND DISCUSSION

Objective 1. A path to commercialization of transgenic rootstocks

a. PIPRA IP analyst, Gabriel Paulino, has served as the main liaison for issues associated with the potential commercialization of transgenic grapevine rootstocks for several CDFA PD/GWSS Board funded projects. He has obtained the necessary APHIS-USDA authorizations to test PGIP-based PD control strategies in vineyards in Solano and Riverside Counties. 'Thompson Seedless' and 'Chardonnay' grapevines expressing the pear fruit PGIP (pPGIP) gene were planted in a jointly operated field trial in Solano County during July, 2010. More details can be found in the report "Field evaluation of grafted grape lines expressing PGIPs" (PI Powell).

b. Grape transformation vector assembly is undergoing re-evaluation.

Objective 2. Identify plant PGIPs that maximally inhibit Xf PG

a. Propagation, grafting and susceptibility testing of grape lines expressing and exporting pPGIP

The transgenic 'Thompson Seedless' and 'Chardonnay' grapevines expressing the pPGIP described in Aguero *et al.* (2005) have been maintained in the UC Davis Core Greenhouse Complex. More individual plants of each cultivar expressing pPGIP and control plants not expressing pPGIP have been rooted with the help of an aeroponic cloner (EZ-Clone, Inc., Sacramento, CA). Details of the grafting procedure are described in the report, "Field evaluation of grafted grape lines expressing PGIPs" (PI Powell).

Collaborator, Victor Haroldsen, has shown that pPGIP protein is found across graft junctions of grapes and tomato plants. That is, it moves from transgenic rootstocks into wild-type tomato scion leaf tissue (**Figure 1**). For these experiments, he used existing stocks of polyclonal pPGIP antibodies after concentrating leaf extract samples 30-fold. Once the monoclonal antibody is available (see report, "Tools to identify PGIPs transmitted across grapevine grafts, PI Powell), its increased specificity will allow for quantification of the amount of pPGIP protein crossing the graft junction into wild-type tissues.

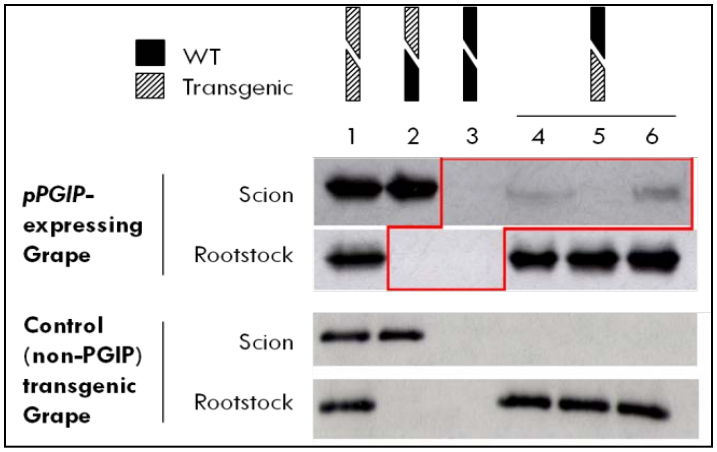

Figure 1. Western blot of leaf extracts taken from rootstock and scion portions of grafted 'Thompson Seedless' grapevines. Transgenic vines are expressing either pPGIP or NPTII (control). pPGIP is visualized crossing from transgenic rootstocks into wild-type (WT) scion tissue (lanes 4-6). This movement is not seen in the reciprocal graft (lane 2).

Testing of the susceptibility of the scion portions of plants to PD has begun using the plants in the field (details in the report "Field evaluation of grafted grape lines expressing PGIPs," PI Powell). Insufficient numbers of scions grafted with pPGIP expressing rootstocks are currently in the field for testing susceptibility, but additional grafted plants are in progress.

b. Selection of PGIPs as PD defense candidates and PGIP-*Xf*PG modeling

Fourteen candidate PGIPs were initially selected for *in vitro* and *in vivo* *Xf*PG inhibition assays based on predicted protein charge and phylogenetic analyses. The homology models created for *Xf*PG, the polygalacturonic acid (PGA) substrate for PG, and each of the candidate PGIPs provided predictive tools to interpret the inhibition mechanisms and physical interactions between *Xf*PG and the PGIPs (Labavitch, 2009). Dynamic *in silico* reaction simulations predicted that two clusters of amino acids, #63-74 and #223-226, must be unblocked for *Xf*PG to cleave PGA. The long columns of electronegative residues on the concave faces of the PGIP's leucine rich repeat structure bind to these critical regions (**Figure 2**). This information coupled with surface chemistry mapping predicts that pPGIP, CsiPGIP (citrus), and OsPGIP1 (rice) will be the best inhibitors of *Xf*PG.

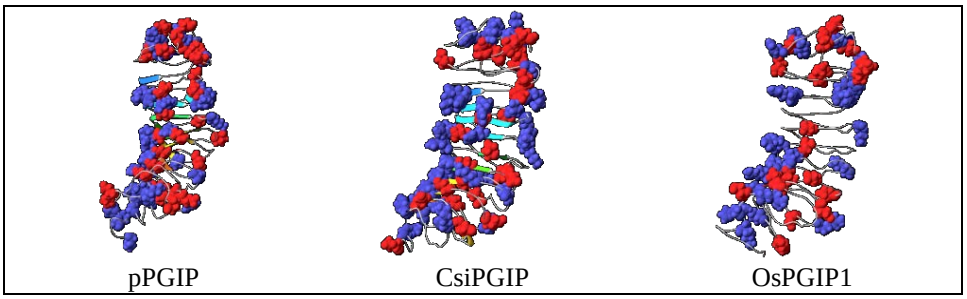

Figure 2. Homology models of three PGIPs predicted to be good candidates to inhibit *Xf*PG. The column of electronegative residues (red) on the concave faces of each protein may align with critical residues on *Xf*PG important for inhibition.

A closer look at the dynamic reaction simulations highlighted other residues that may also influence PG-PGIP binding. Strong hydrogen bonding occurs between residues on pPGIP and Tyr303 of *Xf*PG, bringing them together in a potentially inhibitory manner (**Figure 3**). Electrostatic repulsions between VvPGIP (grape PGIP) residues and *Xf*PG Tyr303 prevent a similar alignment and may predict a failure to inhibit *Xf*PG. Combining modeling predictions and future inhibition data will allow us to evaluate the predicted interactions and infer other potentially useful interactions between the candidate PGIPs and other PGs.

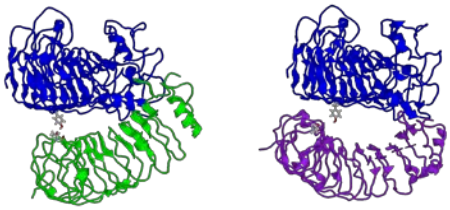

Figure 3. *Xf*PG-PGIP complexes. Tyr303 of *Xf*PG (blue) binds strongly with a region of pPGIP (green) which is not possible with VvPGIP (purple). Interactions such as this might influence PG-PGIP interaction and inhibition.

We are hoping to add unpublished PGIP sequences from non-vinifera *Vitis* varieties to model in the future. These sequences will be obtained as part of a collaboration, currently in negotiation, with a research group at Stellenbosch University, South Africa. The sequences are the property of an industry board associated with the Institute for Wine Biotechnology at Stellenbosch University. It will be of interest to determine how the models of these non-vinifera PGIPs compare to the modeled structure of VvPGIP from *Vitis vinifera* cv. 'Pinotage.'

Based on these modeling studies the two PGIPs (from rice and citrus) have been selected for further study of their inhibition of the PG produced by *Xf*. PCR primers for the amplification and cloning of the PGIP sequences from citrus and rice have been designed (**Table 1**) and are being tested. Genomic DNA has been prepared from rice and citrus and PCR reactions using these and other primers has begun.

Table 1.

Primer sequence*	Primer name	Gene amplified; Modifications
TCACagatcttccatggATGAGtAACACGTCA	CsiPGIP_F3	CsiPGIP; BglII and NcoI sites, alternate frame nonsense mutation
TTCAAAccATGgGCAACACGTCACTG	CsiPGIP_Falt2	CsiPGIP; NcoI site, S2G missense mutation
CCAGgctagcgcgaccctcaatTCTTTC	CsiPGIP_R3	CsiPGIP; NheI site, Xa site, removal of TGA
ccatggtATGCGCGCCATGGTaGTC	OsPGIP1_F	OsPGIP1; NcoI site, alternate frame nonsense mutation
ccATGgGCGCCATGGTCGT	OsPGIP1_Falt	OsPGIP1; NcoI site, R2G missense mutation
gctagcgcgaccctcaatATTGCAG	OsPGIP1_R	OsPGIP1; NheI site, Xa site, removal of TAA
cgagatctccATGGATGTGAAGCTCCTG	OsPGIP2_F2	OsPGIP2; BglII and NcoI sites
gctagcgcgaccctcaatTCGACGAC	OsPGIP2_R3	OsPGIP2; NheI site, Xa site, removal of TAA

*Uppercase bases are homologous to the reference sequence; lowercase bases are introduced changes.

c. *Xf*PG expression and purification

Two strategies have been developed to express active *Xf*PG to use to evaluate the PGIPs. First, an *Xf*PG expression system utilizing Drosophila S2 cells was developed to provide active, stable *Xf*PG protein for *in vitro* inhibition assays. The cloning strategy fused the coding sequence of *Xf*PG to a C-terminal polyhistidine tag for purification and an N-terminal targeting sequence for extracellular secretion of the protein (Labavitch, 2009). Media from transiently transfected cells induced to express *Xf*PG has a small amount of PG activity, as shown by radial diffusion assay (**Figure 4**; Taylor and Secor, 1988). *Xf*PG was partially purified from the medium and pelleted Drosophila cell lysate and analyzed by Western blotting and Coomassie staining SDS-PAGE. Putative *Xf*PG bands, cross-reacting with a tagged antibody recognition site on the recombinant protein, were visualized at 78 kDa in Western blots for cell lysate preparations (**Figure 5**). The protein bands in the cell medium preparation eluant were visualized at 68 kDa by Coomassie staining (**Figure 6**). Each of these preparations showed very slight PG activity, as measured by reducing sugar analysis (Gross, 1982). These activities, however, diminished over time.

The second strategy was to express *Xf*PG transiently in leaves. We have successfully cloned the *Xf*PG into pCAMBIA1301 and introduced this construct into *A. tumefaciens* for transient expression in tobacco leaves. The design of the vector is shown in **Figure 7**. To insure the extracellular localization of the *Xf*PG protein, the protein coding sequence was modified so that the pPGIP extracellular targeting sequence was linked to the 5' end of the *Xf*PG coding sequence. PGIPs are naturally targeted to the apoplast probably as a result of this targeting sequence. We anticipated that the fusion construct pPGIP::*Xf*PG would yield more obvious infiltration results than the unmodified native *Xf*PG construct because the pPGIP signal sequence has been shown to target proteins to the extracellular space. Thus, by targeting the pPGIP::*Xf*PG protein to the cell apoplastic space, it can degrade the pectin-rich middle lamellae and cell walls to simulate the situation in infections and also be inhibited by any co-infiltrated PGIP in or PGIP efficacy tests. It

has been reported that the infiltration assay will work on grape leaves, so the constructs will also be tested in grape leaves. However, the final pPGIP::*Xf*PG construct contained a single base change due to a PCR error that resulted in no active protein.

The advantage of the leaf infiltration assay is that it should be quicker than testing Arabidopsis lines expressing *Xf*PG and PGIPs so we have focused more on generating the material for testing in tobacco.

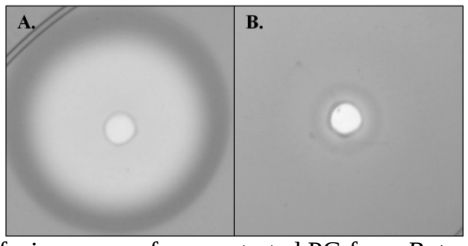

Figure 4. Radial diffusion assay of concentrated PG from *Botrytis cinerea* (A) or culture media from induced *Xf*PG-expressing Drosophila cells (B). The clearing zone diameter is related to amount of PG activity.

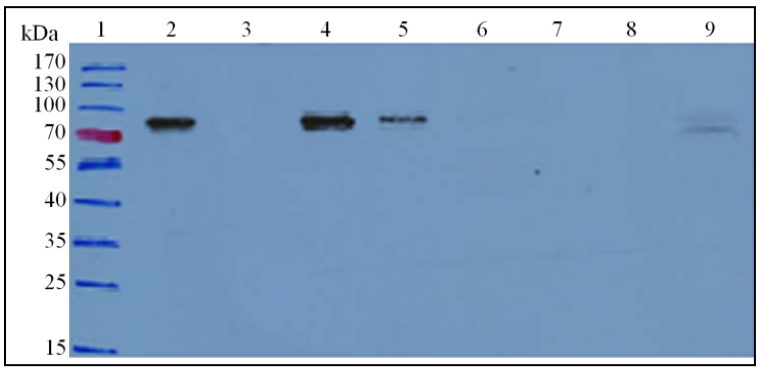

Figure 5. Western blot analysis of partially purified cell lysate after *Xf*PG protein expression. 15 mL crude *Xf*PG lysate was purified by column chromatography and selected fractions were analyzed by Western blotting. Lane 1 = pre-stained ladder, lane 2 = flow-through #4, lane 3 = wash #10, lanes 4-7 = elution fractions #1-4, lane 8 and 9 = cellular medium. Recombinant *Xf*PG protein was eluted with 250 mM imidazole and probed with the anti-V5 primary antibody and anti-mouse HRP secondary antibody.

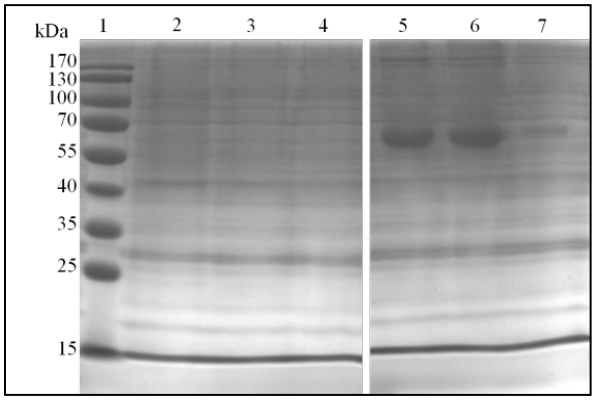

Figure 6. Partially purified *Xf*PG protein eluted with 250 mM imidazole. Coomassie stained polyacrylamide gel electrophoresis. Lane 1 = pre-stained ladder, lanes 2-4 = cell lysate fractions #1-3, lanes 5-7 = cellular medium fractions #1-3.

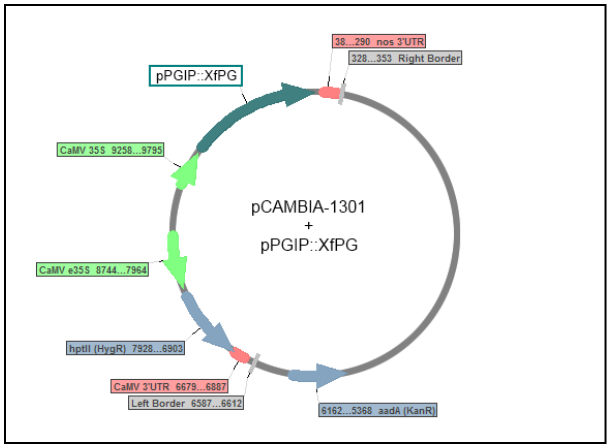

Figure 7. Transient *Xf*PG expression vector for agroinfiltration in tobacco leaves.

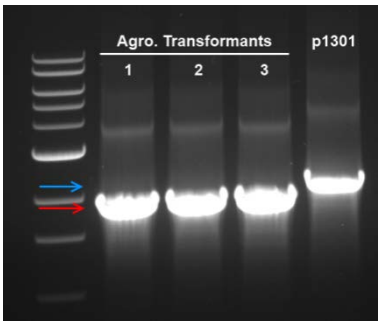

Figure 8. The DNA gel image shows the PCR products from *Agrobacterium* plasmid DNA. Three colonies containing the *Xf*PG:pPGIP sequence (1-3) were screened with p1301 primers flanking the insert site. The expected size fragment is 1924 bp (red arrow). The last lane used "empty" p1301 (gusA intact) as a control template and the same p1301 primers resulted in the gusA product with the expected size of 2204 bp (blue arrow).

d. Expression of PGIPs in *Arabidopsis* and tobacco for *Xf*PG inhibition assays

The previously reported strategies for cloning each of the 14 candidate PGIPs into pCAMBIA-1301 and transformation into *Agrobacterium tumefaciens* (EHA105 pCH32) continues (**Table 2**; Labavitch, 2009).

The *Xf*PG expression construct (**Figure 7**) provides a potential diagnostic tool to test the efficacy of each PGIP *in planta* using a tobacco leaf infiltration system. It has been reported that the infiltration assay will work on grape and tomato leaves and as this approach provides advantages in terms of time and cost, we will continue to develop and use this technique for testing the inhibition of PGs by different test PGIPs. Co-infiltration of *Agrobacterium* cultures harboring *Xf*PG and either pPGIP or LePGIP in pCAMBIA-1301 was carried out as described by Joubert *et al.* (2007). Fully formed leaves of *Nicotiana benthamiana* and *N. tabacum* were infiltrated with constant manual pressure using a needle-less syringe, forcing bacterial cultures into the abaxial leaf tissue. In most cases, initial infiltration zones were marked on the adaxial surface and had measured areas of approximately 35 mm^2. Visual symptom development was observed at 24 and 72 hours post infiltration (hpi, **Figure 9**). Infiltration with cultures expressing *Xf*PG resulted in marked wilting, localized water soaking, and chlorotic lesions developing in the infiltration zone. Leaves co-infiltrated with *Xf*PG and PGIP expressing cultures displayed attenuated symptoms while leaves infiltrated with just PGIP or empty vector cultures showed no symptoms. LePGIP (tomato PGIP) was less effective than pPGIP at inhibiting wilting and lesion development when co-infiltrated with *Xf*PG. Further work to quantify the results will provide a measure of the inhibition of *Xf*PG by each cloned PGIP. We anticipate that the fusion construct pPGIP::*Xf*PG will yield more easily scored results due to the targeted delivery of the *Xf*PG to the apoplast.

Table 2. Cloning progress chart. Checkmarks indicate completed checkpoints while circles indicate work in progress.

Protein (Organism)	Cloning Progress Checkpoints				
	Source tissue acquired	PGIP cDNA isolated	Transformed into *E. coli*	Transformed into *Agrobacterium*	Plant transformation
AtPGIP1 (Arabidopsis)	✓	✓	✓	O	-
AtPGIP2 (Arabidopsis)	✓	✓	✓	O	-
BnPGIP1 (Rapeseed)	✓	✓	O	-	-
CaPGIP (Pepper)	✓	O	-	-	-
CsiPGIP (Orange)	✓	O	-	-	-
FaPGIP (Strawberry)	✓	✓	O	-	-
OsPGIP1 (Rice)	✓	✓	O	-	-
OsPGIP2 (Rice)	✓	✓	O	-	-
PvPGIP2 (Bean)	✓	✓	O	-	-
PpePGIP (Peach)	O	-	-	-	-
PfPGIP (Firethorn)	✓	O	-	-	-
pPGIP (Pear)	✓	✓	✓	✓	✓
LePGIP (Tomato)	✓	✓	✓	✓	O
VvPGIP (Grape)	O	-	-	-	-
*Xf*PG (*Xylella*)	✓	✓	✓	✓	✓
pPGIP::*Xf*PG	✓	✓	✓	O	-

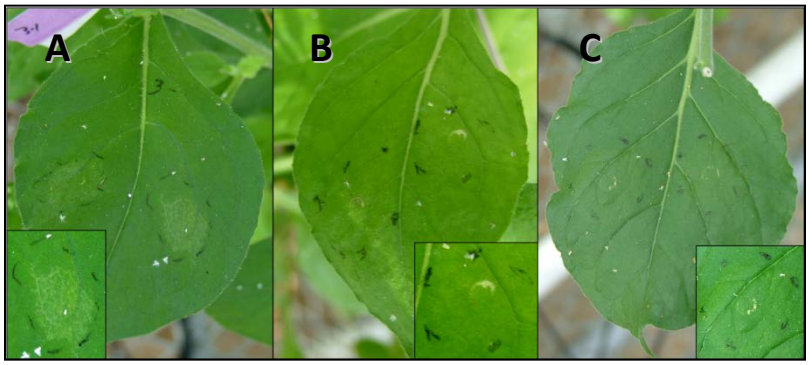

Figure 9. Transient expression of *Xf*PG, pPGIP, and LePGIP in *N. benthamiana* leaves by infiltration with *Agrobacterium* cultures. Chlorotic lesions and water soaking mark the site of agro-infiltrations with *Xf*PG (A). Symptoms are reduced when *Xf*PG is co-infiltrated with pPGIP (B) or LePGIP expressing *Agrobacterium* (C). Inserts show details of infiltration sites. Black marks indicate the borders of the initial zone infiltrated.

e. Modeling of PGIP:*Xf*PG interactions is covered under B above.

Objective 3. Maximize PGIP expression in and transport from roots

The transformation vector to be used in grape transformation has been reevaluated for its effectiveness. Information pertaining to potential signal sequences targeting PGIPs to xylem tissues for transport to and across graft junctions into wild-type scions has been reported by the project "*In planta* testing of signal peptides and anti-microbial proteins for rapid clearance of *Xylella*" (PI: A. Dandekar).

Objective 4. No activity for this reporting period as the optimal PGIP has not been evaluated *in planta.*

CONCLUSIONS

The comparisons of multiple PGIPs are key steps in advancing the use of transgenic rootstocks for PD control in commercial applications. Homology models of all 14 candidate PGIPs have been constructed and critical residues for *Xf*PG-PGIP interaction were discovered. Recombinant *Xf*PG, produced from transiently transfected Drosophila cells, was purified and shown to have a low level of PG activity. Further work to clone and express the candidate PGIPs continues. A more efficient assay, a co-infilitration assay on tobacco leaves, has been developed to assess PGIP inhibition of *Xf*PG. Grape leaves will be tested for their suitability for this assay. *In planta* co-infiltration assays have shown that both pPGIP and LePGIP are able to inhibit the chlorotic lesion development in tobacco leaves that is caused by *Xf*PG-harboring *Agrobacterium*. The ability of one of the candidate PGIPs discussed here, pPGIP, to provide PD resistance to wild-type scions is currently being determined by the field trials.

The overall goal of the project is to develop transgenic grape rootstock lines that express PGIPs that effectively reduce the virulence of *Xf*, an approach that should help to solve the PD/GWSS problem. The project is designed to identify specific PGIPs that optimally inhibit the virulence factor, *Xf*PG, and to express these PGIPs in grape rootstocks to provide PD protection in scions. The expression of PGIPs will utilize transformation components with defined intellectual property (IP) and regulatory characteristics, as well as expression regulating sequences that result in the maximal production of PGIPs in rootstocks and efficient transport of the proteins through the graft junctions to the aerial portions of vines so that *Xf* movement is limited in infected scion tissues. We have modeled 14 candidate PGIPs to predict how they physically interact with *Xf*PG and to combine this knowledge with *in vitro* and *in planta* assay results measuring the ability of each candidate PGIP to inhibit *Xf*PG. For these inhibition assays we are developing separate systems to generate high levels of active *Xf*PG and PGIPs. The best inhibiting PGIPs will be expressed in test grape rootstock germplasm and, after grafting, their ability to limit PD development in non-transgenic scions will be determined.

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